Meningococcal Outer Membrane Vesicle Vaccine Given Intranasally Can Induce Immunological Memory and Booster Responses without Evidence of Tolerance

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We have studied the ability of outer membrane vesicle (OMV) vaccines from Neisseria meningitidis serogroup B to induce vaccine-specific antibody and spleen cell proliferative responses in mice after being administered intranasally (i.n.) and/or subcutaneously (s.c.). A series of four weekly i.n. doses (25 μg) without adjuvant or a single s.c. dose (2.5 μg) with aluminum hydroxide was followed 2 months later by secondary i.n. or s.c. immunizations. After i.n. priming, both immunoglobulin G (IgG) antibody responses in serum, measured by enzyme-linked immunosorbent assay, and IgA antibodies in saliva and extracts of feces were significantly boosted by later i.n. immunizations. The IgG antibody responses in serum were also significantly augmented by secondary s.c. immunization after i.n. as well as s.c. priming. Sera from mice immunized i.n. reached the same level of bactericidal activity as after s.c. immunizations. The s.c. immunizations alone, however, had no effect on mucosal IgA antibody responses, but could prime for booster antibody responses in secretions to later i.n. immunizations. The i.n. immunizations also led to marked OMV-specific spleen cell proliferation in vitro. Both serum antibody responses and spleen cell proliferation were higher after i.n. priming and later s.c. immunizations than after s.c. immunizations alone. There was thus no evidence that i.n. priming had induced immunological tolerance within the B- or T-cell system. Our results indicate that a nonproliferating meningococcal OMV vaccine given i.n. can induce immunological memory and that it may be favorably combined with similar vaccines for injections.

Most pathogens enter the host through the mucosal membranes where the immunological processes are initiated. Vaccines administered directly onto mucosal surfaces are intended to mimic these processes, which include a mucosal immune response, characterized by secretory immunoglobulin A (IgA) antibody production not normally induced by parenteral vaccines. Mucosal vaccines consisting of live, attenuated microbes have indeed been shown to effectively induce mucosal as well as systemic immune responses of importance for protection against disease (27). However, since live vaccines may themselves carry some risk of disease, several research groups have focused on the development of nonreplicating mucosal vaccines (18, 26). Results of animal experiments suggest that such vaccines based on microbial components may be effective only if a so-called mucosal adjuvant is added (18). Serious concerns have been raised, however, that nonliving protein material delivered onto mucosal surfaces may induce a state of tolerance (12, 15). It has also been shown during the last few years that even cholera toxin (CT) or its B-subunit, which are strong mucosal adjuvants for induction of antibody responses, may actually be tolerogenic when it comes to T-cell-dependent immunity (14, 24).

We have been able to show that formulations of bacterium-derived particles can induce both local mucosal and systemic antibody responses when applied to various mucosal surfaces of mice (2, 6, 11, 13). With a heat-killed whole-cell pneumococcal vaccine, however, a far better effect was obtained when administered intranasally (i.n.) than when given into either the oral cavity, the stomach, or via rectum into the lower intestine (13). It was also evident that i.n. immunizations with simple suspensions in saline of particles derived from pneumococci, serogroup B streptococci, Bordetella pertussis, and serogroup B meningococci induced very good antibody responses, even without the addition of CT (2, 6, 11, 13). It thus appeared that these particles possessed some kind of self-adjuvanticity.

The immunogenicity of such nonreplicating particles was confirmed by a limited study in humans immunized i.n. with an outer membrane vesicle (OMV) vaccine prepared from group B meningococci (9). The functional ability of the serum antibodies, measured in this study as bactericidal activity, was moreover better than expected from the antibody concentrations measured by conventional enzyme-linked immunosorbent assay (ELISA). It also became evident that the same vaccinees responded with an increase in vaccine-specific T-cell proliferation (21). Simple formulations of particles derived from airway pathogens may therefore represent a model system for development of efficient nonreplicating nasal vaccines.

In the present study, we have focused on the development in mice of immunological memory and the possibility that i.n. or subcutaneous (s.c.) priming with a meningococcal OMV vaccine might lead to booster responses to later i.n. or s.c. immunizations.
nizations. The aim was furthermore to determine whether intranasal immunizations might be combined with parenteral immunizations and to evaluate the question of immunological tolerance.

MATERIALS AND METHODS

Animals. Inbred female BALB/c mice, 8 to 10 weeks old, were obtained from Bomholtgård Breeding and Research Center, Ry, Denmark.

Vaccine preparation. The s.c. vaccine contained OMVs from the epidemic group B meningococcal strain 44/76 (15:P1.7,16) adsorbed onto Al(OH)₃. The OMVs were prepared by extraction of bacteria with 0.5% deoxycholate in 0.1 M Tris-HCl buffer (pH 8.6) containing 10 mM EDTA and purified by differential centrifugation (7). Each s.c. dose of 200 μl consisted of 2.5 μg of OMVs measured as protein. The nasal vaccine was prepared from the original pool of OMVs used in the s.c. vaccine formulation, but without Al(OH)₃. Each nasal dose of 30 μl consisted of 25 μg of OMVs measured as protein.

Immunizations. In order to study antibody responses to OMVs, groups of eight mice were immunized either i.n. four times at weekly intervals with 25 μg of OMVs, without Al(OH)₃, or s.c. with a single dose of 2.5 μg of OMVs with Al(OH)₃ as adjuvant. The s.c. dose was given at the same time as the second i.n. dose. Two months later, these mice were given a second course of either four i.n. immunizations or a single s.c. dose. Groups of previously unimmunized control mice were then given primary immunizations via the i.n. or s.c. routes. The i.n. immunizations were carried out by holding the mice in a supine position with the head down while 30 μl of the antigen solution was delivered slowly with a micropipette onto the nares so that the mouse could sniff it in. The mice were briefly anesthetized intravenously with 0.01 ml (10 mg/ml) of propofol (Diprivan; Zeneca Ltd., Macclesfield Cheshire, United Kingdom) before i.n. immunization, after which they recovered completely within 1 to 2 min. The s.c. vaccine was given without anesthesia.

For the study of primary cellular immune responses to OMVs, groups of 18 mice each were immunized four times i.n. or once s.c., as in the antibody study, whereas one group of nonimmunized mice served as controls. This would allow for the collection of spleens from groups of six mice at three different time points (see below). In order to measure spleen cell proliferative responses to secondary s.c. immunizations, groups of 36 mice were immunized either i.n. or s.c. or not immunized, as described above. This was followed 3 months later by a secondary s.c. dose given to half (18 mice) of the mice in each group, whereas the other half (18 mice) were left unimmunized.

Collection of samples. In both the first and second courses of immunizations to evaluate antibody responses, saliva, feces, and serum were collected before and 1 week after the fourth i.n. dose, corresponding to 3 weeks after the s.c. dose. Saliva was collected with absorbent wicks consisting of synthetic fibers and cellulose (Polyfiltronics Group, Inc., Rockland, Mass.) after a single intraperitoneal injection of 0.1 mg of pilocarpine-HCl (Sigma Chemical Co., St. Louis, Mo.) in 200 μl of phosphate-buffered saline (PBS), and the net weight was recorded. Two wicks saturated with saliva were obtained from each mouse, frozen at −20°C in 1.5-ml microcentrifuge tubes, and subsequently extracted with 400 μl of PBS with 0.05% Tween 20 and protease inhibitors, as described previously (8). Three to five pieces of freshly voided feces were collected into 1.5-ml microcentrifuge tubes, frozen at −20°C, and subsequently vacuum dried in a Speed Vac Concentrator (Savant Instruments, Inc., Farmingdale, N.Y.), before their net dry weights were recorded. Extracts of feces were made by homogenization of feces, as described previously (8). The final volumes were adjusted to 200 μl/well. After 6 days of incubation in 5% CO₂ at 37°C, the cells were pulsed with [³H]thymidine (1.3 μc/sample) (Amer sham, Little Chalfont, United Kingdom) for 18 h and harvested (Packard Instrument). Incorporated [³H]thymidine was determined by liquid scintillation counting (Packard TopCount). Proliferative spleen cell responses were expressed as the reciprocal of the mean triplicate cpm value for the antigen in triplicate wells.

Statistical analyses. The significance of differences between groups of animals was determined by the two-tailed Mann-Whitney U test with PRISM software (GraphPad Software, San Diego, Calif.).

RESULTS

Immunizations i.n. can prime for and boost mucosal and systemic antibody responses. After the first series of four i.n. doses of the OMV vaccine, significant IgG and IgA antibody responses, measured by ELISA, were reached, respectively, in serum (median value, 522 kU/ml) (Fig. 1, upper panel) and saliva and feces (medians, 3.5 kU/ml and 1.1 kU/g, respectively) (Fig. 2 and 3, upper panels). The concentrations of antibodies persisted until the next series of four weekly i.n. vaccine doses, after which the concentrations were significantly augmented compared with the responses after primary immunizations alone (serum IgG median, 1,202 kU/ml, P = 0.015; saliva IgA median, 10.9 kU/ml, P = 0.003; feces IgA median, 3.6 kU/g, P = 0.007). The i.n. secondary immunizations could...
thus boost both serum and mucosal antibody concentrations that had been induced by i.n. priming.

In serum, the IgG antibody responses were also significantly augmented by secondary s.c. immunization (median, 2,793 kU/ml), compared to responses after i.n. priming only \((P = 0.0002)\) (Fig. 1, upper panel). These responses to secondary s.c. immunizations did not differ significantly from the responses to secondary i.n. immunizations \((P = 0.2)\). On the other hand, the IgA antibody concentrations in saliva and feces were not influenced by the secondary s.c. immunizations after i.n. priming (Fig. 2 and 3, upper panels). Thus, mucosal immunizations seemed to be necessary to obtain mucosal antibody responses.

Immunizations s.c. can prime for, but not boost, mucosal antibody responses. Primary s.c. immunizations alone induced serum IgG antibody responses (median, 414 kU/ml), but no effect on IgA antibodies could be detected in saliva or feces (Fig. 1 to 3, lower panels). In serum, secondary immunizations by both the i.n. and s.c. routes led to significant booster responses (medians, 6,077 and 2,926 kU/ml, respectively) compared to responses induced by s.c. priming alone \((P = 0.0012\) and \(P = 0.0003\), respectively) (Fig. 1, lower panel). With these immunization regimens, it appeared that i.n. boosting was just as effective as s.c. boosting following priming via the s.c. route \((P = 0.3)\).

Surprisingly, i.n. secondary immunizations induced strong IgA antibody responses in both saliva and feces after s.c. priming (medians, 8.1 kU/ml and 22.4 kU/g) (Fig. 2 and 3, lower panels). These responses were not different from those obtained after i.n. priming \((P > 0.2)\) and indicate that s.c. immunizations can effectively prime the mucosal immune system for later booster responses to mucosal immunizations. On the other hand, secondary immunizations by the s.c. route did not lead to any measurable mucosal antibody responses. This is in line with the previous observation that mucosal immunizations were of importance for obtaining sizable antibody responses at the mucosal surfaces, whether the mucosal immune system had been primed by s.c. or by mucosal immunizations.

The i.n. immunizations induced serum bactericidal activity at the same level, as did s.c. immunizations. Sera from mice that were primed i.n. showed bactericidal activity in the same range as those from mice primed s.c. \((P = 0.3)\) (Table 1). In the i.n. primed group, secondary s.c. immunizations induced significant further increases in bactericidal activity \((P = 0.015)\), whereas secondary i.n. immunizations did not. Significant increases in bactericidal titers were also observed in sera from mice that were primed s.c. and boosted i.n. or s.c. \((P = 0.009\) and \(P = 0.04\), respectively). After secondary immunizations, there was no significant difference \((P = 0.2)\) in bactericidal titers of the group that had been primed and boosted i.n. from the group that had received s.c. immunizations only.

Immunizations i.n. can prime spleen cells for vaccine-specific in vitro proliferation. The spleen cell response of i.n. vaccinated mice increased with each immunization, and 4 weeks...
after start of the weekly i.n. immunizations, proliferation was significantly higher than in the control group ($P < 0.009$) (Table 2). In mice immunized once s.c. with adjuvant, proliferation was of the same magnitude as after three i.n. doses without adjuvant (week 3) ($P = 0.6$).

Three months after the primary series of four i.n. OMV vaccine doses (Fig. 4, second column), antigen-specific spleen cell proliferation was still significantly increased ($P = 0.0001$) compared to the corresponding response obtained with non-immunized control mice (Fig. 4, last column). This response to i.n. immunizations was significantly higher ($P = 0.02$) than those obtained 3 months after a primary s.c. immunization (Fig. 4, third and fifth columns) had a higher background level (cpm without antigen) than the other groups. In part, this might explain the observation that mice that had been primed and boosted s.c. had lower response levels than the group that had only been primed s.c. Immunizations i.n. may not induce immunological tolerance. Antibody responses in serum were stronger after i.n. priming and secondary s.c. immunizations (median value, 2,793 kU/ml) (Fig. 1, upper panel) than after priming only via the s.c. route (median, 414 kU/ml) (Fig. 1, lower panel, left). It was thus evident that i.n. immunizations had a positive ($P = 0.001$) rather than a tolerogenic effect on the systemic antibody response.

TABLE 2. Vaccine-specific spleen cell proliferation in groups of six mice after primary i.n. or s.c. immunizations with meningococcal OMV vaccines

<table>
<thead>
<tr>
<th>Wk</th>
<th>Median Δcpm (range) for immunizationa</th>
<th>Controls</th>
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<tbody>
<tr>
<td>2</td>
<td>13,740 (0–84,780) 3,027 (0–6,412)</td>
<td>0 (0–8,972)</td>
</tr>
<tr>
<td>3</td>
<td>24,460 (838–40,680) 20,580 (0–28,750)</td>
<td>4,935 (0–14,470)</td>
</tr>
<tr>
<td>4</td>
<td>67,700 (8,293–187,800) 17,180 (0–37,160)</td>
<td>2,467 (0–14,350)</td>
</tr>
</tbody>
</table>

a Four i.n. doses were given at weekly intervals, starting at week 0, and one s.c. dose was given at week 1. A group of unimmunized mice served as controls. Spleen cells were collected at weeks 2, 3, and 4 and stimulated for 6 days in vitro with OMVs. Δcpm, cpm minus background values obtained in the absence of OMVs.
sponses to later s.c. immunization. Similarly, vaccine-specific spleen cell proliferation was higher in the group that had been primed i.n. and immunized s.c. later than in all other groups that had been immunized s.c. only (P < 0.004) (Fig. 4). Even when the effect of spontaneous proliferation or background activity was not subtracted for, the influence of i.n. immunizations on later responses to s.c. immunizations was still positive (P < 0.005).

DISCUSSION

A vaccine consisting of OMVs from group B meningococci suspended in saline has previously proved to be strongly immunogenic in mice and humans when administered i.n. as drops or spray, even without the use of additional mucosal adjuvants (6, 9, 21). The results of the present study with mice showed that i.n. immunizations with such OMV vaccines could also prime the immune system for both mucosal and systemic booster antibody responses to later repeated i.n. immunizations. This is in agreement with previous observations that mucosal immunizations may lead to immunological memory (17). Moreover, the booster antibody responses in serum to secondary i.n. immunizations without adjuvant were just as prominent as those after s.c. immunizations with adjuvant, whether the priming had been made i.n. or s.c. Also when testing for serum bactericidal activity, i.n. immunizations were just as effective as s.c. immunizations. Thus, it appears that formulations of nonproliferating mucosal vaccines may be favorably combined with traditional injectable vaccines, at least when it comes to systemic antibody responses.

When comparing the antibody responses to vaccines given by different routes, the higher dose of the nasal vaccine compared to the s.c. vaccine has to be taken into account. One reason for this was the increased waste and spillage of nasal drops compared to an injected formulation; another was the lack of additional adjuvant of the nasal vaccine. A small amount of deoxycholate in the OMV preparations might also have an effect on mucosa different from that in the s.c. tissue, although a mucosal vaccine consisting of whole heat-inactivated meningococci devoid of deoxycholate was just as immunogenic as the OMVs. Ongoing experiments indicate, however, that the number of nasal vaccine doses might actually be reduced if time is allowed for immunologic memory to develop (H. Bakke, unpublished observations).

As expected, mucosal antibody responses in the present study were not seen after s.c. immunizations alone. It was surprising, however, that high levels of vaccine-specific antibodies were found in both saliva and feces after s.c. priming followed by i.n. boosting. This finding indicates that the secretory and systemic immune systems are not totally segregated and confirm previous observations that it is possible to prime intestinal immune responses by parenteral immunizations (see reference 3 for review). It may also help explain the observation that mucosal immunizations can induce antibodies at mucosal areas distant from the induction site (4, 8, 13). Although the most prominent antibody responses are found in secretions close to the sites of induction (4, 8, 9), the impact of s.c. priming on intestinal antibody responses to i.n. immunizations in this study is further in favor of a “common” or “regionalized” mucosal immune system (4, 19). At any rate, this observation might be of advantage in the design of future immunization regimes to improve an anticipated protective effect at the mucosal level.

The marked proliferation of spleen cells from mice that had received the OMV vaccine i.n., when exposed to the vaccine antigens in vitro, indicates that T cells had also been primed in vivo by these immunizations. This is in line with previous observations showing an in vitro vaccine-specific proliferation of peripheral blood mononuclear cells from humans who had received a similar vaccine i.n. (21). The pronounced increases in proliferation in the present study with the first series of weekly repeated i.n. immunizations suggest that immunological memory might have been induced as well within the T-cell system.

The in vitro spleen cell proliferation in the mice that had received the OMV vaccine s.c. was surprisingly low compared to what was achieved with i.n. immunizations and was not in accordance with the findings in humans, in which the proliferation of peripheral blood mononuclear cells was more marked after parenteral than after i.n. immunizations (20, 21). In the present study, the low degree of spleen cell proliferation could in part be explained by a concomitant high background activity (i.e., the spleen cells obtained shortly after s.c. immunizations proliferated spontaneously in vitro without the addition of vaccine antigens). It seemed also that the in vivo priming of spleen cells by s.c. immunizations had a different kinetic pattern than that seen after i.n. immunizations.

Normally, soluble proteins may induce a state of immunological tolerance when administered onto mucosal surfaces (16). However, we found no evidence that the particulate protein vaccine used in this study had any negative effect on antibody responses upon later exposure to the same antigens via the i.n. or s.c. routes. In fact, it seemed that these responses were not at all hampered by preformed local mucosal or systemic antibodies. Mucosal vaccines may therefore have an advantage over parenteral vaccines that may be less effective in individuals who have preexisting antibodies (e.g., in infants with systemic IgG antibodies derived prenatally) (25).

Previous observations by others have indicated that mucosal vaccines consisting of soluble proteins may also lead to substantial antibody responses, especially when mucosal adjuvants are added, but at the same time to induction of tolerance within the T-cell system (12). Similarly, feeding of CT, which is known as a strong antibody inducer, may result in specific T-cell tolerance when later injected (14). Moreover, the use of the B-subunit of CT as a mucosal adjuvant for antigens fed to test animals has been found to induce T-cell tolerance on later exposure to that antigen (5). In the present study with the OMV vaccine, however, i.n. priming augmented rather than reduced the vaccine-specific spleen cell proliferation in response to later s.c. immunizations. In our hands, therefore, the OMV vaccine did not induce any sort of immunological tolerance. It is tempting to speculate that the reason for this might be the similarity of the bacterium-derived particulate vaccine to the infectious agent itself and the fact that the vaccine is deposited at a mucosal surface to which the proper infectious agent will also attach and colonize. Our results suggest that nonproliferating vaccines based on bacterium-derived particles may be effective when given i.n. and that they may well be used in conjunction with similar vaccines for injection.
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